Static mechanical stretching accelerates lipid production in 3T3-L1 adipocytes by activating the MEK signaling pathway

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Shoham N, Gottlieb R, Sharabani-Yosef O, Zaretksy U, Benayahu D, Gefen A. Static mechanical stretching accelerates lipid production in 3T3-L1 adipocytes by activating the MEK signaling pathway. Am J Physiol Cell Physiol 302:C429–C441, 2012. First published October 19, 2011; doi:10.1152/ajpcell.00167.2011.—Understanding mechanotransduction in adipocytes is important for research of obesity and related diseases. We cultured 3T3-L1 preadipocytes on elastic substrata and applied static tensile strains of 12% to the substrata while inducing differentiation. Using an image processing method, we monitored lipid production for a period of 3–4 wk. The ratio of %-lipid area per field of view (FOV) in the stretched over nonstretched cultures was significantly greater than unity (P < 0.05), reaching ~1.8 on average starting from experimental day ~10. The superior coverage of the FOV by lipids in the stretched cultures was due to significantly greater sizes of lipid droplets (LDs) with respect to nonstretched cultures, starting from experimental day ~10 (P < 0.05), and due to significantly more LDs per cell between days 10 and ~17 (P < 0.05). The statically stretched cells also differentiated significantly faster than the nonstretched cells within the first 10 days (P < 0.05). Adding peroxisome proliferator-activated receptor-γ (PPARγ) antagonist did not change these trends, as the %-lipid area per FOV in the stretched cultures that received this treatment was still significantly greater than in the nonstretched cultures without the PPARγ antagonist (14.44 ± 1.96% vs. 10.21 ± 3%; P < 0.05).

Hence, the accelerated adipogenesis in the stretched cultures was not mediated through PPARγ. Nonetheless, inhibiting the MEK/MAPK signaling pathway reduced the extent of adipogenesis in the stretched cultures (13.53 ± 5.63%), bringing it to the baseline level of the nonstretched cultures without the MEK inhibitor (10.21 ± 3.07%). Our results hence demonstrate that differentiation of adipocytes can be enhanced by sustained stretching, which activates the MEK signaling pathway.

mechanotransduction; obesity; tensile strain; 3T3-L1 preadipocytes; differentiation

OBEITY IS ONE OF THE MAJOR preventable causes of morbidity and mortality, and its prevalence is rising in adults as well as in children (16, 29, 53). There are numerous reports in the literature regarding the contribution of obesity to the risks for developing type 2 diabetes, hypertension, atherosclerosis, and hyperlipidemia (29, 51); this motivates research regarding the etiology of obesity, which, at a cell scale, importantly involves characterization of specific stimuli that promote synthesis of cytosolic lipids.

Adipose tissue is a complex organ, which, other than storing energy in the form of triglycerides (which is essential for metabolism of glucose), is able to secret hormones and cytokines that regulate systemic metabolism and homeostasis (9, 29, 51, 76). The increased adipose tissue mass in obesity is due to hypertrophy and hyperplasia of adipocytes. The number of adipocytes can increase through mitosis (mostly in the preadipocyte phase) or through differentiation of additional preadipocytes. It is currently unclear which of these two pathways actually functions, or is more dominant in vivo, but it is often surmised that maturation of preadipocytes into adipocytes is one of the most important mechanisms in obesity (1, 9, 10, 18, 29, 51, 76).

Conservative treatment of obesity is generally aimed at decreasing the mass of excessive adipose tissues by changing the balance between calorie intake and expenditure, via physical exercise, diet control, or both. Current understanding of cellular mechanotransduction, however, might suggest that there is more to obesity than energy imbalance. Specifically, the responsiveness of cells to their mechanical loading environment was already demonstrated for many cell types (22–24, 36, 78); osteoblasts, for example, produce mineralized bone given a sufficient (dynamic) mechanical stimulation (5, 36, 66). Adipocytes in weight-bearing adipose tissues that are exposed to static large tensional and compressive mechanical strains in vivo (47, 68) might be mechanosensitive and mechanically responsive as well. Using a combined MRI and finite element modeling approach, Linder-Ganz and colleagues (47, 48) found that, during normal sitting, adipose tissues at the buttocks are subjected to tensile strains that are as high as 30% (averaged across subjects in their study); a lying posture induces tensile strains that are approximately half that magnitude. Hence, given that adipocytes are subjected to substantial tensile strains physiologically at weight-bearing adipose tissues, it is important to determine whether sustained tensile strains are promoting adipogenesis or not, in the context of a sedentary lifestyle, obesity, and adipose-related diseases.

Here we hypothesize that sustained static mechanical loading of preadipocytes can influence the adipogenesis process (i.e., the differentiation response) in these cells. Dynamic loading modes such as cyclic stretching or vibration were generally found to suppress adipocyte differentiation in cell cultures of different developmental stages, e.g., preadipocytes or mesenchymal stem cells that originate from adipose tissue or bone marrow (8, 12, 35, 61–63, 72–74, 77). While the effects of dynamic loading have been extensively investigated (8, 12, 35, 61–63, 72–74, 77), there has been little investigation into the effects of static loading on adipogenesis. To our knowledge, the influence of static loading on differentiation of adipocyte cultures was examined in only two studies so far (27, 32). Specifically, static loads appeared to have a dual effect on adipogenesis, where static stretching applied to mature hyper-
trophic adipocytes accelerated differentiation (27) but static compression (32) impeded it. Nevertheless, these studies (8, 12, 27, 32, 35, 61–63, 72–74, 77) were limited by the methodology of destructive testing of lipid contents in the cultures, which was done by chemical staining (Oil Red O), Western blotting, and reverse transcription polymerase chain reaction (RT-PCR) (8, 12, 27, 32, 35, 61–63, 72–74, 77). Destructive testing limits the number of time points at which the cells can be studied and compromises the statistical power since cultures cannot be used as their own controls. Most importantly, there was no study so far that looked at the effects of static mechanical loading at the preadipocyte stage, where cells were not yet differentiated, and hence, the question of whether the differentiation process in adipocytes itself could be regulated by mechanical loading needs to be addressed.

In the present work we cultured preadipocytes on elastic substrata and applied sustained static tensile strains to the substrata while inducing differentiation in the cultures. Using our new nondestructive image processing-based method for measuring the numbers and sizes of lipid droplets (LDs) in maturing adipocytes (57), we monitored the lipid production (adipose conversion) in the cultures every 2–3 days for about 3–4 wk. In addition, we examined the effects of mitogen-activated protein kinase kinase/mitogen-activated protein kinase (MEK/MAPK) inhibitor and peroxisome proliferator-activated receptor (PPAR)-γ antagonist on adipogenesis in stretched and nonstretched cultures. Our present findings are relevant to research of obesity and adipose-related diseases as well as to adipose tissue engineering.

**METHODS**

**Cell culturing.** We cultured preadipocytes as described previously (56, 57). Specifically, mouse embryonic 3T3-L1 cells (American Type Culture Collection) were cultured in a growth medium (GM) consisting of high-glucose Dulbecco’s modified Eagle’s medium (DMEM, 4.5 mg/ml; Biological Industries, Israel), 10% fetal bovine serum (Biological Industries), 1% l-glutamine (Biological Industries), 0.1% penicillin-streptomycin (Pen-Strep; Sigma, Israel), and 0.5% 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES; Sigma). The maximum confluence allowed for the cultures was ~80% before passages. Differentiation was induced by changing the GM to a differentiation medium (DM). The DM consisted of the aforementioned GM, supplemented with 5 μg/ml insulin (Sigma), 1 μM dexamethasone (Sigma), and 0.5 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma). Three days after induction of differentiation, the DM was replaced by a supporting medium (SM) consisting of the GM supplemented with 5 μg/ml insulin (Sigma). The SM was changed every 2–3 days.

**Cell-stretching apparatus.** We selected to load the preadipocytes in a stretching mode based on our previous work, which showed that tissue compression associated with weight-bearing postures involves coupled, substantial stretching of the cell bodies (47, 48, 68). Accordingly, we built an apparatus for applying equibiaxial and homogenous tensile strain distribution in weight-bearing adipose tissues during sitting and lying (47, 48). We deliberately selected to deliver constant, continuous mechanical loading effects (loading effects).

The 3T3-L1 preadipocytes were cultured according to the protocol described in **Cell culturing**. Next, 10^5 cells were seeded per each well in a six-well BioFlex collagen-coated culture plate (Flexcell). When a confluence of ~90% was achieved, the culture plate was mounted in the cell-stretching apparatus, and three wells were subjected to stretch, whereas the other three wells were used as nonstretched control cultures. Differentiation was then induced in all the wells as described above in **Cell culturing**. The cells used for the experiments were always younger than passage 13. We monitored the cultures during periods of 18–28 days (until cultures became too confluent for adequate monitoring). Cultures were inspected almost daily and a normal differentiation process was always observed. Every 2–3 days CO_2. Accordingly, the dimensions of the apparatus were 20 × 11 × 6 cm. The bottom frame of the apparatus was made of polycarbonate, and the other parts were made of Stainless Steel 303. The apparatus was sterilized using 70% ethanol (Bio Lab) before culturing.

We verified and characterized the homogeneous and biaxial strain state in the substrata (4, 36, 71) as follows. When the elastic substrata were in the undeformed configuration, we photographed three marks that were previously drawn on the substrata. Then, the top frame was lowered to a measured distance from the bottom plate (d) and we photographed the marks again in the deformed configuration. These images were processed by a MATLAB code (MathWorks), and the three Eulerian strain components (E_t, E_s, and E_0) were calculated for each d using the finite strain theory, as detailed previously (5, 20, 45). The two normal strain components, E_t and E_s, are the strains along the circumferential and radial axes, respectively, whereas E_0 is the shear strain component. We calculated these stain components for d in a range of 0.83–1.51 cm, which are the minimum and maximum d allowed by the apparatus. The average increment in d was 0.08 cm. We repeated this calibration experiment five times and calculated the mean and standard deviation of each strain component (Fig. 2A). After verification of an equibiaxial strain state (E_t = E_s, E_0 = 0) by means of a two-way analysis of variance (ANOVA) for the factors of strain component and d (P < 0.05), the effective tensile strain E was calculated as the average of E_t and E_s (Fig. 2B). We fitted the plot of E versus d with a 2nd-order polynomial function (Pearson correlation coefficient = 0.98):

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E(d) = -0.28d^2 + 0.47d - 0.08
\]

We verified that the substrata for cell culturing in the BioFlex plates are linear elastic by means of uniaxial tensile tests, where we stretched three specimens of the substrata (14 cm long, 2 cm wide) at a rate of 5 mm/min (Instron 5544 model, High Wycombe, UK). The substrata demonstrated a linear elastic behavior up to a strain magnitude of 18%.

Additionally, we conducted viscoelastic creep experiments to ensure that strains in the substrata do not increase significantly over time under the sustained loads. For this purpose, we calculated the three Eulerian strain components in the substrata immediately after applying the maximal deformation (E = 12%) and after 24 h. We repeated this experiment five times as well (Fig. 2C). Given that the mean and standard deviation of each strain component occurred shortly after the load was applied (i.e., within several hours) and were negligible thereafter, we concluded that creep effects could be ignored since changes in the strains, even after 24 h, were within the accuracy of strain measurements (Fig. 2C).

**Experimental design.** Using the cell-stretching apparatus described above in **Cell-stretching apparatus**, we subjected six triplicates of 3T3-L1 cultures to substrate tensile strains of 12%, which is an intermediate value with respect to normal physiological tensile strain distributions in weight-bearing adipose tissues during sitting and lying (47, 48). We deliberately selected to deliver constant, continuous strains (rather than intermittent loading) over the entire culturing period to determine the effects of static loading per se on lipid production in the cultures (that is, without any masking from dynamic loading effects).

The 3T3-L1 preadipocytes were cultured according to the protocol described in **Cell culturing**. Next, 10^5 cells were seeded per each well in a six-well BioFlex collagen-coated culture plate (Flexcell). When a confluence of ~90% was achieved, the culture plate was mounted in the cell-stretching apparatus, and the load was applied to the substrata and induced differentiation in the cultures. Differentiation was then induced in all the wells as described above in **Cell culturing**. The cells used for the experiments were always younger than passage 13. We monitored the cultures during periods of 18–28 days (until cultures became too confluent for adequate monitoring). Cultures were inspected almost daily and a normal differentiation process was always observed. Every 2–3 days
we digitally photographed the cultures under a phase contrast microscope (Eclipse TS100, Nikon) at an original magnification of ×40 using a field of view (FOV) of 220 × 165 μm² to optically document the progress of adipose conversion. Photographs were always taken without removing the culture plates from the cell-stretching apparatus; hence stretched cultures remained stretched during the visualization as well. Three different FOVs were considered in each well, and at least five cells were captured in each micrograph. We analyzed the micrographs using our previously developed nondestructive adipocyte-micrograph image processing method. The validation studies of our image processing method for monitoring intracytoplasmic triglycerides, which are specific markers for the adipose conversion (60), were reported in our previous work (57). In brief, Or-Tzadikario et al. (57) compared diameters of 90 randomly selected and automatically measured LDs with manual measurements of the same LDs, obtained using the SigmaScan Pro software (Systat Software), which is commonly employed for measuring entities in micrographs (57). That paper reported that the agreement between the automatic and manual measurements was very good (57), and therefore it was concluded that the image processing-based technique was adequate for monitoring the development of LDs over time. Additionally, examples of our image processing algorithm implemented on Oil Red O- and Nile Red-stained adipocytes (56) versus nonstained adipocyte cultures (Figs. 3 and 4) demonstrate the ability of our image processing technique to correctly identify the locations and sizes of intracytoplasmic lipid droplets. Oil Red O specifically stains intracytoplasmic triglycerides by dissolving in the lipoid substance (60), and Nile Red detects intracellular LDs under fluorescence microscopy by fluorescing in the presence of triglycerides (26). Hence, these comparisons provide additional visual verification of our presently used image processing-based nondestructive technique. In the image processing algorithm, each micrograph was converted into a binary image, in which only LDs were in white and everything else was blackened (Fig. 4). Then, we manually marked cells in each micrograph (5 cells per each micrograph). Our MATLAB code (provided in full in the Appendix of Ref. 57) automatically calculated the percentage area covered by LDs, the number and diameter of LDs, and the number of differentiating cells in a FOV. Subsequently, we calculated the ratio of each of these adipogenesis parameters in the stretched cultures over the nonstretched cultures in the same six-well plates. The above experiment was repeated six times, and descriptive statistics of all the aforementioned parameters (means and standard deviations) were calculated for the entire data set from the six trials. Finally, we compared each property ratio to unity (unity indicates that stretching
had no effect on adipogenesis) using ANOVA for the factor of time postinduction of differentiation. $P < 0.05$ was considered statistically significant.

Measurements of triglyceride content and leptin expression. We conducted Oil Red O staining studies to compare between lipid contents in stretched versus nonstretched cultures at day 17 postinduction of differentiation. The staining protocol was previously described (60). In brief, a working solution of Oil Red O was prepared by dissolving 4.2 g of Oil Red O (Sigma) in 1,200 ml absolute isopropanol. Subsequently, we added 900 ml of double-distilled water (DDW) to the solution and left it overnight at 4°C. Triplicates of stretched and nonstretched cultures were fixed for 10 min with 4% paraformaldehyde, then washed with phosphate-buffered saline, stained for 1 h by complete immersion in a working solution of Oil Red O and carefully rinsed with DDW. The stained cultures were then digitally photographed. These micrographs were analyzed by a red color threshold: each red pixel in the red-blue-green micrograph was converted into a white pixel and everything else was blackened. Furthermore, 1 ml of isopropanol was added to the stained cultures, and the absorbance of the extracted dye was immediately monitored spectrophotometrically at 510 nm (SpectraMax 340PC384, Molecular Devices). The Oil Red O absorbance between the stretched and nonstretched cultures was compared using a nonpaired t-test.

We further conducted alkaline phosphatase (ALP) histochemistry staining studies since ALP activity and fat storage were found to be tightly linked during 3T3-L1 differentiation (2). Specifically, triplicates of nonstretched versus stretched cultures were fixed in immersion of 0.3% paraformaldehyde for 1 h. Diazonium salt solution was prepared by dissolving 25 mg of Fast Blue RR salt in 48 ml DDW. Naphthol AS-MX phosphate alkaline (2 ml) was then added to the solution. The reagent mixture was incubated with the fixed culture cells for 30 min in room temperature. Cultures were then rinsed thoroughly in DDW.

In addition, we examined the extent of leptin expression, being one of the most abundant adipocytokines (55), in the stretched versus the nonstretched cultures. For this purpose, we used a Mouse Leptin ELISA kit (ab100718; Abcam), which is an in vitro enzyme-linked immunosorbent assay for quantitative measurement of mouse leptin in culture supernatants. The media in triplicates of stretched and nonstretched cultures were sampled at days 4 and 17 postinduction of
determination, and the amount of leptin was measured using the ELISA kit according to the manufacturer’s instructions. We compared the concentration of the expressed leptin between the stretched and nonstretched cultures, using a nonpaired \( t \)-test.

**Examining the roles of PPAR\(\gamma\) and MEK signaling.** We examined the effects of PPAR\(\gamma\) antagonist and MEK inhibitor on the adipogenesis in the stretched and nonstretched cultures, as follows. Six triplicates of 3T3-L1 preadipocyte cultures were grown on elastic substrata as described above in *Cell culturing*. When a confluence of \( \sim 90\% \) was achieved, differentiation was induced in all the wells (as again described in *Cell culturing*). The substrata in three triplicates were then subjected to 12% strain. A selective and irreversible PPAR\(\gamma\) antagonist GW9662 (Calbiochem) or MEK/MAPK inhibitor (PD98059; Calbiochem) was added to the DM in one triplicate of the stretched preadipocyte cultures and in one triplicate of the nonstretched preadipocyte cultures at a concentration of 10 \( \mu \)M and 50 \( \mu \)M, respectively; these concentrations were previously shown to be highly specific to the PPAR\(\gamma\) transcription factor and the MEK/MAPK pathway, respectively, in 3T3-L1 cells (3, 14, 15, 31, 38, 42, 43, 58). Control cultures received dimethyl sulfoxide as a vehicle. The DM was changed every 2–3 days. Given that in our preliminary experiments we observed stretched-induced accelerated lipid production at day 17 postinduction of differentiation (Fig. 5), we digitally photographed and analyzed the cultures at that time point. The micrographs were processed as described above in *Experimental design*, and the following adipogenesis parameters were calculated: 1) percentage lipid area per FOV, 2) mean diameter of LDs per cell, 3) mean number of LDs per cell, and 4) mean number of differentiated cells per FOV. The data were analyzed by means of the Mann-Whitney statistical test using SPSS software (version 17, IBM). Differences were considered to be significant when \( P < 0.05 \).

**RESULTS**

**Adipogenesis.** Example comparisons between micrographs of stretched and nonstretched cultures at different experimental time points are provided in Fig. 4 (processed images are shown at the *top right* frames). The progress of adipose conversion was well observed in both the stretched and nonstretched cultures, but LDs in the stretched cultures (Fig. 4, *right*) became substantially greater than in the nonstretched cultures (Fig. 4, *left*) at corresponding time points after \( \sim 10 \) experimental days. Consequently, larger cells were typically observed in the stretched cultures (Fig. 4). These example micrographs are visual evidence for the accelerated lipid production in the stretched adipocyte cultures starting from the second week of the experiments.

Quantitative analyses of our experimental data are provided in Fig. 5. The ratio of %-lipid area per FOV in the stretched over nonstretched cultures was significantly greater than unity \( (P < 0.05) \) and reached a value of \( \sim 1.8 \) on average for the period between 8 days after induction of differentiation plus stretching and the end of experiments. The superior coverage of the FOV by LDs in the stretched cultures over that period was due to significantly greater sizes of LDs with respect to nonstretched cultures starting from approximately day 10 postinduction of differentiation and stretching (\( P < 0.05 \); Fig. 5B), as well as due to the existence of significantly more LDs per cell between days 10 and 17 (\( P < 0.05 \); Fig. 5C). Specifically, on the last week of experiments (i.e., starting at about day 21), LD diameters in adipocytes belonging to the stretched cultures were at least \( \sim 1.4 \) times greater than LDs in the nonstretched cultures (Fig. 5B). At day 28 postinduction of differentiation, LD diameters in the stretched cultures were \( \sim 1.8 \) times greater than in the nonstretched cultures. The peak value of the ratio of mean number of LDs per cell in stretched over control cultures, being \( \sim 1.8 \), occurred approximately at day 15 postinduction of differentiation and stretching (Fig. 5C). Interestingly, the statically stretched cells also differentiated significantly faster than the cells in the nonstretched cultures within the first \( \sim 10 \) days of the experiments (Fig. 5D, \( P < 0.05 \)). For example, on experimental days 8 and 10 there were on average \( \sim 2.1 \) times and \( \sim 1.8 \) times greater numbers of differentiating cells (i.e., cells which contained identifiable LDs) per FOV in the stretched cultures than in the nonstretched cultures, respectively \( (P < 0.05; \text{Fig. } 5D) \). However, at the portion of the time course starting around experimental day 13 and lasting until experiments terminated, cells in the stretched and nonstretched cultures differentiated at a statistically indistinguishable rate (Fig. 5D).

**Measurements of triglyceride content and leptin expression.** Micrographs of nonstretched versus stretched cultures stained...
with Oil Red O, 17 days postinduction of differentiation, and the corresponding processed micrographs presenting only the red pixels are provided in Fig. 6. These processed images (Fig. 6, right) provide additional visual verification of our image processing-based method as a tool for identifying intracytoplasmic triglycerides. In addition, the accelerated adipogenesis in the stretched cultures can clearly be observed (Fig. 6). Consistent with our results that were obtained using the image processing algorithm, the Oil Red O absorbance measured in the stretched cultures (0.28 ± 0.02 optical density) was significantly greater than in the nonstretched cultures (0.23 ± 0.05 optical density) (Fig. 6C) (P < 0.05).

Representative micrographs of nonstretched versus stretched cultures stained for ALP activity are provided in Fig. 7, A and B, respectively. Differentiated adipocytes and the accumulated LDs can be observed in dark gray and white, respectively. Stained cells indicated activity of ALP. Consistent with the Oil Red O staining results, we visually observed greater ALP activity in the stretched, compared with the nonstretched cultures, which further supports our findings that adipogenesis is accelerated in the stretched cultures.

Leptin concentrations measured at days 4 and 17 postinduction of differentiation in the stretched versus the nonstretched cultures are presented in Fig. 8. At day 4 postinduction of differentiation in the same six-well plate and same experimental session, taken 14 (A), 18 (B), and 25 (C) days postinduction of differentiation and stretching. Top right frames show the corresponding black and white processed images for quantitative data analysis (see METHODS). Scale bars represent 50 μm.
differentiation, the leptin concentration in the stretched cultures was 1.23 times higher than in the nonstretched cultures, but this difference was not statistically significant (Fig. 8). Nonetheless, at day 17 postinduction of differentiation, the leptin concentration in the stretched cultures was significantly higher than that in the nonstretched cultures, being 3.73 ± 0.32 versus 3.38 ± 0.28 pg/ml ($P < 0.05$) (Fig. 8). Given that leptin expression was found to be positively associated with the sizes of adipocytes (67), and the latter positively correlates with the differentiation stage of the adipocytes (65), these findings provide additional evidence that the lipid production process was enhanced in the stretched cultures.

Examining the roles of PPARγ and MEK signaling. The adipogenesis parameters calculated in stretched and nonstretched cultures with and without MEK inhibitor are presented in Fig. 9. When comparing between stretched cultures treated with the MEK inhibitor and stretched cultures that did not receive this treatment, we found that the %-lipid area per FOV was statistically indistinguishable (Fig. 9A). Likewise, when comparing nonstretched cultures treated with the MEK inhibitor to nonstretched cultures without treatment, %-lipid area per FOV was indistinguishable (Fig. 9A). Differences in %-lipid area per FOV came out as significant (Fig. 9A; 16.34 ± 4% vs. 10.2 ± 1.4%; $P < 0.05$) when comparing stretched versus nonstretched cultures that were not treated with the MEK inhibitor (which is consistent with our previous results), but when comparing stretched cultures that received the MEK inhibitor treatment to nonstretched cultures that did not receive the treatment, there was no statistically significant difference again. In this latter case, even though LDs were significantly smaller in the stretched cultures with MEK inhibitor (Fig. 9B; 5.51 ± 1.1 μm) compared with the nonstretched cultures without the MEK inhibitor (Fig. 9B; 6.58 ± 1.67 μm) ($P < 0.05$), significantly more differentiated cells per FOV (Fig. 9D;
24.39 ± 8.89 vs. 14.98 ± 4.97; *P < 0.05) produced approximately the same amount of lipids in the stretched cultures with MEK inhibitor and in the nonstretched cultures without the MEK inhibitor. In other words, inhibition of the MEK/MAPK signaling pathway reduced the extent of adipogenesis in the stretched culture group, bringing it to the baseline level of the nonstretched group without the MEK inhibitor.

The adipogenesis parameters calculated in stretched and nonstretched cultures with and without PPARγ antagonist are presented in Fig. 10. Adding PPARγ antagonist to the nonstretched cultures reduced the % lipid area per FOV from 10.2 ± 3% to 7.6 ± 3.5% (Fig. 10A), and in the stretched cultures, the PPARγ antagonist reduced the % lipid area per FOV from 16.34 ± 4% to 14.4 ± 2% (Fig. 10A), but both decreases were not statistically significant. Even though LDs were significantly smaller in the stretched cultures with the PPARγ antagonist compared with the stretched cultures without the PPARγ antagonist (Fig. 10B; 6.8 ± 1.5 vs. 8.26 ± 2.35 μm, respectively; *P < 0.05), greater numbers of LDs per cell (Fig. 10C; 15 ± 10.7 vs. 12 ± 7.4) and greater numbers of differentiated cells per FOV (Fig. 10D; 16.15 ± 6.4 vs. 14.7 ± 3.2) both contributed to the similar coverage of the FOVs by
lipids. Most importantly, the % lipid area per FOV in the stretched cultures, either with or without the PPARγ antagonist, was significantly greater than in the nonstretched cultures without the PPARγ antagonist (Fig. 10A; 10.21 ± 3%) (P < 0.05). In the case of the stretched cultures without the PPARγ antagonist, this latter observation was manifested by significantly greater LDs (Fig. 10B; 8.26 ± 2.3 vs. 6.58 ± 1.66 μm) (P < 0.05). In the case of the stretched culture with the PPARγ antagonist, the superior coverage of the FOV by lipids, compared with the nonstretched cultures without PPARγ antagonist, was attributed to 1) greater sizes of LDs (Fig. 10B; 6.8 ± 1.6 vs. 6.58 ± 1.66 μm); 2) greater numbers of LDs per cell (Fig. 10C; 15 ± 10.8 vs. 11.6 ± 7); and 3) more differentiated cells per FOV (Fig. 10D; 16.15 ± 6.14 vs. 14.9 ± 5). In other words, the PPARγ antagonist did not suppress the accelerated lipid production in the stretched cultures.

DISCUSSION

The present study provides clear evidence that static stretching stimulates adipocyte cultures to produce lipids at a faster rate. Our results show that both the larger numbers and greater sizes of LDs contributed to the consistently superior coverage of the FOV by lipids in the stretched cultures, starting at
approximately day 10 postinduction of differentiation and stretching. These experimental data hence support our hypothesis presented in the Introduction that adipocytes are mechano-sensitive and mechanoresponsive and that the adipose conversion process is influenced by mechanical stimulation.

Adding PPARγ antagonist reduced the %_lipid area per FOV in both the stretched and nonstretched cultures, which is consistent with knowledge regarding the regulation of adipogenesis by PPARγ (21, 50, 75). However, in the present study, this antagonist did not suppress the mechanoinduced accelerated lipid production observed in the stretched cultures. It is recognized that the PPARγ transcription factor is involved in the initial phase of cell differentiation, but once cells are on the adipocyte-phenotype pathway, the functionality of PPARγ is less or not at all influential. Thus, a downstream effector should play a role in this process, and hence we examined the MEK/MAPK pathway and we did find that inhibiting this pathway had a significant influence on suppressing the accelerated adipogenesis in stretched cultures. Indeed, the MAPK signaling pathway was previously found to be mechanically activated in various cell types (e.g., vascular endothelial cells, human fibroblasts, and mesenchymal stem cells) and in response to different mechanical loading regimes (e.g., shear stresses, tensile loading, and static or dynamic hydrostatic pressures) (37, 41, 49, 54). Importantly, consistent with our present results, Tanabe and colleagues (72) also found that the extracellular signal-regulated kinases (ERK) system was mechanically activated in 3T3-L1 preadipocytes (considering that ERK is activated by the MEK). Nevertheless, in the study of Tanabe and colleagues (72), activating the ERK/MAPK pathway resulted in inhibition of adipogenesis in the stretched cultures, which is the opposite type of effect reported here. Indeed, the ERK/MAPK was found to have both inhibitory (17, 33, 39, 64) and stimulatory (7, 40, 52, 58, 79) effects on adipose conversion. Considering that Tanabe and colleagues used a different mechanical loading regime (their cells were cyclically loaded to 120% or 130% of their undeformed length at a frequency of 1 Hz for 15 or 45 h), and given that stimulation of the ERK/MAPK pathway might have opposing effects in the process of adipogenesis depending on the time of activation during the differentiation process (57, 71), either inhibition or acceleration of the adipogenesis could be expected. In a recent paper, Hara and colleagues (27) examined the effects of static stretching of up to 120% in mature 3T3-L1 adipocytes that were exposed to the stretch for 72 h. Rho-Rho kinase was found to be activated and contribute to the accelerated adipogenesis in the stretched adipocytes. The different pathways that were found to be involved in our present study and in the Hara et al. study could be a result of different durations of stretching (72 h vs. 17 days), different developmental stages of the stretched cells (mature adipocytes vs. preadipocytes), and different loading modes (20% static uniaxial strain vs. 12% equibiaxial strain). Interestingly, even though two different signaling pathways were found to be activated by mechanical stretching (MEK/MAPK in our study, and Rho-Rho kinase in the Hara study), both pathways show the same influence of accelerating the adipogenesis in stretched cultures.

There is a debate in the literature regarding the role of the ERK/MEK pathway in insulin signaling, as there are studies which report that activation of ERK/MEK stimulates insulin signaling (28) but others reported lack of influence (44) or an inhibitory effect (6, 19). Specifically, the accelerated lipid production observed in the stretched cultures could be an outcome of enhanced insulin signaling by the MEK pathway, as suggested by Harmon and colleagues (28), who showed that MEK inhibitors impair insulin-stimulated glucose uptake in 3T3-L1 adipocytes. On the other hand, this phenomenon might be driven by other mechanotransduction pathways as well, such as mechanosensitive channels, in which gating is initiated in response to mechanical stimuli, resulting in catalyzed transport of glucose across the plasma membrane (34, 58). In this case, insulin signaling could be unaffected, as suggested by Lazar and colleagues (44), who found that MEK inhibition does not block stimulation of glucose utilization by insulin. The work of Fujishiro and colleagues (19) suggests that insulin signaling could even be inhibited when the ERK pathway is activated, as they found that ERK markedly suppresses the expressions of insulin receptor (IR) and its major substrates (IRS proteins) as well as that of the glucose transporter GLUT4. Likewise, Bost et al. (6) concluded that the IRS-1 protein is phosphorylated by the ERK pathway and that this serine phosphorylation exerts an inhibitory effect on the insulin signaling. Now that the present study points to involvement of the MEK pathway in accelerated adipogenesis in stretched adipocytes, future research should focus on the interactions between MEK and insulin signaling in such stretched cells.

The understanding that adipocytes respond to mechanical loading and thus the latter can be used to regulate their differentiation response and control their lipid production is consistent with knowledge regarding mechanotransduction in other cell types, including cardiomyocytes, vascular endothelial cells, smooth muscle cells, skeletal muscle cells, and osteoblasts, which are all responsive to mechanical loads (22–24, 36, 78). Moreover, given that adipocytes originate from the same progenitor cells as do osteoblasts and myocytes, that is, they all normally mature from mesenchymal stem cells (11), it is only reasonable to expect that mechanical signals would influence adipocytes as well. Our present findings clearly indicate that a static (sustained) stretch accelerates lipid production in adipocytes, which could theoretically correspond, for example, to the increased adipose mass in the buttocks of obese individuals, where soft tissue stretching during weight bearing is substantial (13, 48, 70). Contrarily, cyclic stretches delivered at physiological frequencies appear to inhibit lipid production, which is rather intuitive given that physical exercise would generally involve cyclic loading of soft tissues, and since exercise is associated with a decrease in fat tissue mass over time. Specifically, there are numerous reports regarding the inhibiting effects that cyclic stretches induce, in which the differentiation was examined using Oil Red O staining. Additionally, RT-PCR, Western blotting, and immunofluorescence analyses were conducted for identifying gene and protein expressions which are triggered by such cyclic mechanostimuli (8, 12, 35, 61–63, 72–74, 77). Tanabe and colleagues (72, 73) found that cyclic stretching of 3T3-L1 preadipocytes to 120% or 130% of their undeformed length, at a frequency of 1 Hz, for 15 or 45 h, inhibits differentiation. The inhibition of the adipogenesis was found to be mediated by activation of the ERK/MAPK pathway. Later on, Turner and colleagues (77), who exposed human umbilical cord perivascular cells to equibiaxial cyclic stretch (10% half-sine waves at
a frequency of 0.5 Hz for 24 or 60 h) observed inhibition of adipogenesis, however, through the transforming growth factor-β1/Smad signaling pathway. David and colleagues (12) assessed the differentiation of stretched versus nonstretched bovine mesenchymal stem cells as well as of cells from a C3H10T1/2 pluripotent cell line, and they also found that 300 cycles of elongation per day, to a magnitude of 4,000 μe at a frequency of 1 Hz (over a 2-wk period) inhibited differentiation into adipocytes. Sen and colleagues (8, 61, 62) again documented inhibited differentiation to the adipocyte phenotype in the C3H10T1/2 cell line as well as in marrow-derived mesenchymal stem cells, where cells were cyclically stretched to 2% strain at a rate of 10 cycles per minute, up to 3,600 total cycles (over 5 days). The inhibition of adipogenesis in these studies was reported to occur due to activation of the β-catenin pathway. In a later study, Sen and colleagues (63) delivered the following mechanical loading regimes to C3H10T1/2 cells: 1) low-intensity vibration, which they defined as strains below 0.001% applied at a frequency of 90 Hz; as well as 2) high-magnitude cyclic strains (2%, 0.17 Hz). They observed that two 20-min sessions, 1 or 3 h apart, of either low-intensity vibration or high-magnitude strains, suppressed adipogenesis via activating the β-catenin; adding more loading sessions further increased the suppressive effect. Likewise, Tirkkonen et al. (74) used vibrations to mechanically load human adipose tissue stromal cells. A 14-day period of 3 g peak acceleration at a frequency of 100 Hz, which was applied for 3 h per each experimental day, was similarly found to inhibit differentiation into adipocytes. Consistently, Huang et al. (35) delivered equibiaxial peak strains of 10% to rodent stromal cells at a 0.5 Hz frequency for 48 h and observed inhibition of adipocyte differentiation, but when the peak strain was lowered to 2%, the suppressive effect was significantly milder, or even nonexistent when the peak strain was reduced to the 0.5% level. To summarize, different signaling pathways were found to be mechanically activated following cyclic stretching of adipocytes, preadipocytes, or progenitor cell cultures, and the fact that multiple pathways were identified could be due to the differences in experimental protocols and cell types. Combining our present results that concern static stretching with the aforementioned studies, which all investigated the effect of cyclic stretching (8, 12, 35, 61–63, 72–74, 77), we can suggest that sustained static stretching delivered to adipocytes (within a certain physiological range) can stimulate them to produce lipids in vitro, whereas dynamic (cyclic) stretching is able to inhibit lipid production. Such a potential dual-mechanotransduction response is known to exist in other cell types as well, e.g., in osteoblasts whose activity is stimulated by cyclic loading but depressed under static loading (5, 37), being the opposite of the type of response suggested herein.

Our present results may be the foundation of additional work that should be focused on monitoring the adipose conversion in more physiological conditions, for example, using three-dimensional tissue-engineered adipose constructs exposed to different levels of stretch or to compound loading (e.g., hydrostatic pressures and tensile strains applied simultaneously). The effects of intermittent loading can be studied as well, to determine whether occasional relief periods in the stretching are able to counterbalance the accelerated lipid production. Additionally, it may be worthwhile to study how the adipose conversion process is influenced by different concentrations of insulin in the differentiation medium (57) (e.g., in the context of examining interactions between MEK and insulin signaling) or by different levels of glucose (e.g., in the context of a calorie-rich diet), and these biochemical modifications can in turn be studied in combination with mechanical loading. Such studies may eventually open new research paths, driven by mechanotransduction, to explore mechanisms in obesity and related conditions.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: N.S. and A.G. conception and design of research; N.S., R.G., O.S.-Y., U.Z., and D.B. performed experiments; N.S., D.B., and A.G. analyzed data; N.S., D.B., and A.G. interpreted results of experiments; N.S. and A.G. prepared figures; N.S. and A.G. drafted manuscript; N.S., D.B., and A.G. edited and revised manuscript; N.S., R.G., O.S.-Y., U.Z., D.B., and A.G. approved final version of manuscript.

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